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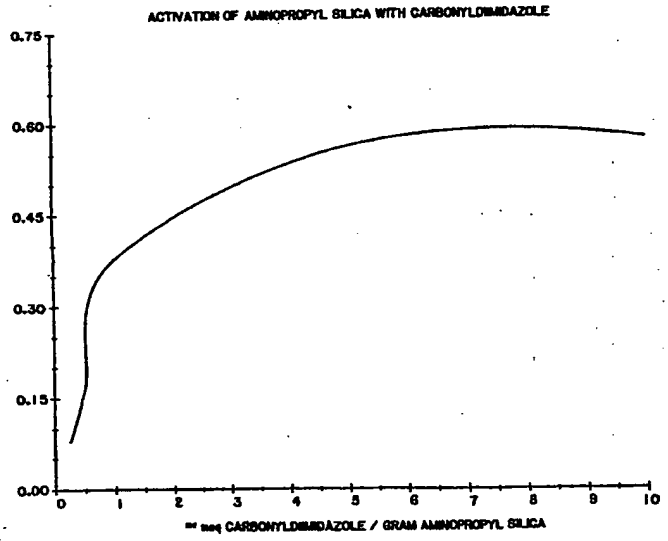
(54) Title: BONDED PHASE CHROMATOGRAPHIC SUPPORTS

(57) Abstract

Functionalization of particulate bonded phase chromatographic supports prepared by silanization of silica gel or controlled pore glass and containing pendant primary alkyl amine groups. Functionalization results from the activation of the amines by reaction with N,N'-carbonyldiimidazole (CDI), or a related azolide, in anhydrous organic solvent, followed by derivatization of the support. Derivatization results from reaction of the activated support with a functionalizing reagent consisting of a primary or secondary, alkyl or aryl amine in organic solvent, or from an aqueous solution of the amine or its salt. A urea linkage results through which the functionalizing reagent is covalently attached t the supp rt. Derivatizati n can result fr m the addition of an excess of a single reagent, or as a consequence of the sequential addition f two or more functi nalizing reagents. Chromatographic supports preparati n in this manner yield materials suitable f r affinity, covalent, ion exchange and hydrophobic interaction chromatography of biom lecules as well as f r the preparation of immobilized reagents.



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Bonded Phase Chromatographic Supports

Field of the Invention

The present invention relates to the field of separation chromatography, and more particularly to bonded phase chromatographic supports of silica or porous glass beads having derivatized supports, and a method of making the same.

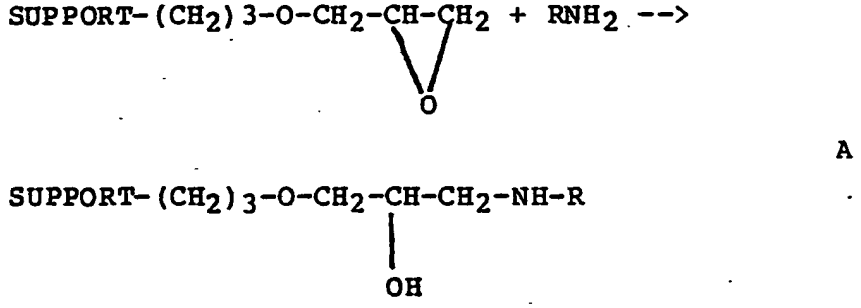
Background of the Invention

Early work in the field of chromatography utilized supports prepared from natural substances such as agarose, cross-linked dextran and the like. These materials, while adequate, lack rigidity, and therefore are not particularly useful for high pressure chromatography because the pressure thereon can deform the substances, or otherwise cause them to collapse, thereby slowing the acceptable flow rate considerably.

More recently, new developments have been made in the field allowing the functionalization of rigid support materials. Among the chromatographic supports used in the separation of biological macromolecules are bonded phase silica and porous glass beads comprising on their surface pendant hydrophilic moeities resulting from reaction of the activated silica or glass surface with a reactive organosilane, immobilizing the organosilane through a siloxane linkage to a silanol group on the surface. The hydrophilic moeity is attached to the reactive organosilane through an alkyl or aryl spacer group. Functional groups including glycidoxypropyl, amino-propyl, polyethyleneoxide and polyethyleneimine and derivatives thereof, have been used.

Certain problems were encountered in using these prior art systems, particularly for chromatographic separation of biological macromolecules. Nonspecific hydrophobic interaction between the straight chain alkyl backbone and the hydrophobic portion of the biological molecules has been observed, particularly when chromatography occurs under aqueous conditions. In addition, ionic interaction and hydrogen bonding has been observed between the silanol matrix and the polar functional groups of the derivatizing silane. This causes the functional groups to double back and bind to the matrix thereby making them unavailable for the intended chromatographic interaction.

In an effort to prepare materials lacking the aforementioned properties, bonded phase chromatographic supports prepared by reaction of 3-glycidoxypropyltrimethoxysilane with activated particulate silica or controlled pore glass were further derivatized by the addition of an amine or other nucleophile to the epoxide functionality of the bonded phase, as shown in Equation A below:



A

Other approaches have included the preparation of phases resulting from the adsorption of polyethyleneimine and polyethyeneoxide to particulate silica followed by derivatization of the polymeric chain.

Derivatives prepared from glycidoxypropyl bonded phases exhibit considerable hydrophobic secondary interactions. Polyether bonded phases exhibit similar problems. These materials require mobile phase additives to mask residual silanol activity and hydrophobic interaction.

Most recently, in an effort to prepare chromatographic support suitable for the separation of biological macromolecules, glycidoxypropyl and methacrylate bonded phases have been copolymerized with acrylamide or functionalized acrylamide.

Summary of the Invention

The invention relates to the functionalization of particulate bonded phase chromatographic supports prepared by silanization of silica gel or controlled pore glass and containing pendant primary alkyl amine groups. Functionalization results from the activation of the amines by reaction with N,N'-carbonyldiimidazole (CDI), or a related azolide, in anhydrous organic solvent, followed by derivatization of the support. Derivatization results from reaction of the activated support with a functionalizing reagent consisting of a primary or secondary, alkyl or aryl amine in organic solvent, or from an aqueous solution of the amine or its salt. A urea linkage results through which the functionalizing reagent is covalently attached to the support. Derivatization can result from the addition of an excess of a single reagent, or as a consequence of the sequential addition of two or more functionalizing reagents. Chromatographic supports prepared in this manner yield materials suitable for affinity, covalent, ion exchange and hydrophobic interaction chromatography of biomolecules as well as for the preparation of immobilized reagents.

The invention relates to the development of bonded phase chromatographic supports wherein the residual silanol activity associated with the particulate silica or controlled pore glass substrate has been effectively masked by application of a hydrophilic barrier. This eliminates the irreversible adsorption of biological macromolecules and low molecular weight amines observed with bonded phase supports which are not further derivatized.

The objectives of the present invention include:

The preparation of a physical barrier preventing interaction between surface silanols and sample components;

The derivatization of the physical barrier preventing interaction between the hydrophobic silane backbone and sample components; and

The functionalization of the physical barrier to impart properties resulting in selective retention of sample components.

In addition, a number of important specific objectives are also achieved using the present invention, including:

The use of N,N'-carbonyldiimidazole for the activation of a chromatographic support with other than pendant hydroxyl groups;

The preparation of a urea derivative of a bonded phase chromatographic support and the unique hydrophilic nature of the urea linkage;

The near quantitative derivatization of bonded supports obtained by this synthetic route;

A multi-step approach wherein the chromatographic support can be prepared to exhibit two or more unique selectivities in a predetermined manner;

The almost infinite variety of ligands which can be employed as functionalizing reagents; and

The effective hydrophilic barrier which results in masking residual silanol activity and the hydrophobic nature of the silane backbone.

Brief Description of the Drawings

The invention may be more fully understood by having reference to the following drawings, wherein:

FIGURE 1 shows the loading of cysteamine obtained, as determined by elemental analysis for sulfur, when varying amounts of N,N'-carbonyldiimidazole were added in THF to aminopropyl silica gel and allowed to react for 3 hours at room temperature, followed by the addition of 2 milli-equivalents of cysteamine in DMSO per gram of activated aminopropyl silica gel. The reaction with cysteamine was allowed to proceed for 24 hours at room temperature.

FIGURE 2 shows the loading of cysteamine and aminothiophenol obtained after 24 hours reaction at room temperature, as determined by elemental analysis for sulfur, when varying amounts of cysteamine or aminothiophenol were added in various solvents to an activated aminopropyl silica gel. The activated aminopropyl silica gel was prepared by reaction of 2 milliequivalents of N,N'-carbonyldiimidazole in THF per gram of aminopropyl silica gel. The activation was allowed to proceed for 3 hours at room temperature.

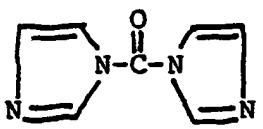
FIGURE 3 shows the loading of cysteamine obtained at various times, as determined by elemental analysis for sulfur, when 2 milliequivalents of cysteamine per gram of activated aminopropyl silica gel were allowed to react for up to 24 hours at room temperature. The activated aminopropyl silica gel was prepared by reaction of 2 milliequivalents of N,N'-carbonyldiimidazole in THF per gram of amin propyl silica gel. The activation was allowed to proceed for 3 hours at room temperature.

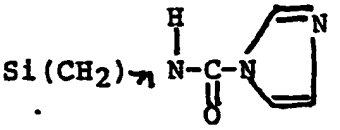
Detailed Invention

The invention relates to the functionalization of bonded phase chromatographic supports prepared by reaction of 3-aminopropyltriethoxysilane, 3-aminopropyltrimethoxysilane, p-(aminomethylaminoethyl) phenethyltrimethoxysilane, aminoethylaminopropyltriethoxysilane, 4-aminobutyldimethylmethoxysilane or 4-aminobutyltriethoxysilane with activated particulate silica or controlled pore glass resulting in a bonded phase chromatographic support bearing pendant primary amine groups, Formula I.

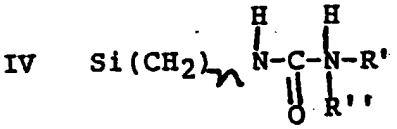
I $Si(CH_2)_n NH_2$

The compound of Formula I is then activated by reaction with N,N'-carbonyldiimidazole (CDI), Formula II, or a related azolide, such as carbonylditriazole, carbonyldibenzimidazole and carbonyldibenztriazole in anhydrous organic solvent including chloroform, methylene chloride, dimethylformamide and dimethylsulfoxide at room temperature to yield a compound of Formula III.

II 

III 

The compound of Formula III is derivatized by reaction with a functionalizing reagent consisting of a primary or secondary, alkyl or aryl amine in organic solvent including chloroform, dimethylformamide and dimethylsulfoxide, or from a buffered aqueous solution of the amine or its salt, at room temperature or at elevated temperature to yield a compound of Formula IV

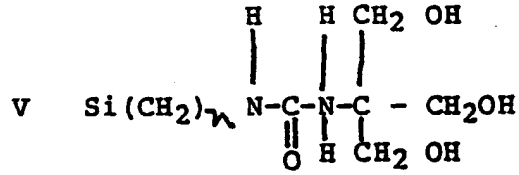
IV 

where R' and R'' = H, alkyl, aryl, or an alkyl aryl.

The urea linkage through which the functionalizing reagent is now covalently attached to the bonded phase support is uncharged under normal chromatographic conditions and provides a hydrophilic barrier masking the properties of the silane backbone and the residual silanol activity beneath it.

If the functionalizing reagent contains an ionic group, for example taurine ($NH_2-CH_2-CH_2-SO_3H$), glycine (NH_2-CH_2-COOH), N,N'-diethylaminoethylenediamine ($NH_2-CH_2-CH_2-N(CH_2CH_3)_2$) or aminoquanidine ($NH_2-NH-C(NH_2)NH$), an ion-exchange chromatographic support will result.

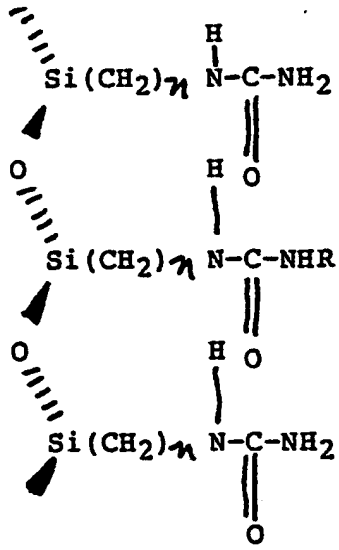
If the functionalizing reagent is tris (hydroxymethyl) aminomethane, a hydrophilic chromatographic support suitable for size exclusion chromatography of biological macromolecules will result, Formula V.

V 

Derivatization of the activated support, Formula III, can result from the addition of an excess of a single functionalizing reagent, or as a consequence of the sequential addition of two or m re functionalizing reagents.

If the functionalizing reagent is a reactive ligand of general formula RNH_2, such as aminophenylboronic acid, aminophenylmercuric acetate or aminophenylarsenoxide, and is added in limited quantity to the activated support of Formula III followed by an excess of ammonia saturated organic solvent, a chromatographic support suitable for covalent chromatography of Formula VI results. Ideally 20-30% of the activated functional groups are derivatized with the reactive ligand. If a greater percentage of derivatization is employed, steric hinderance comes into play reducing the effective capacity of the chromatographic support.

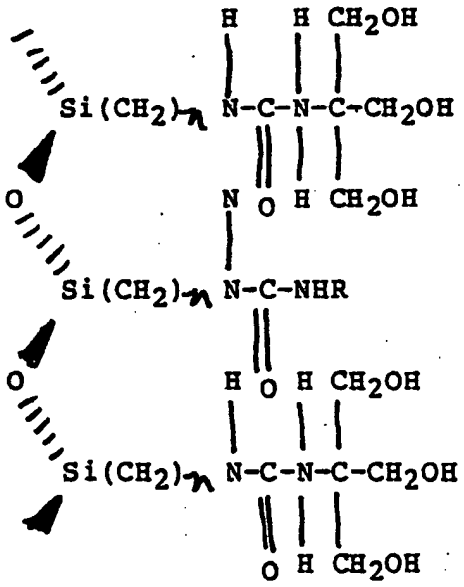
VI



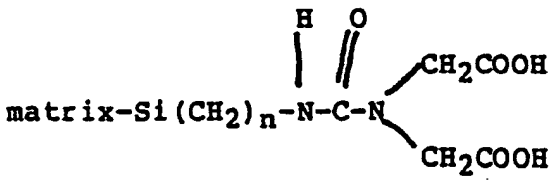
If the functionalizing reagent is an alkyl amine of general formula RNH_2, such as n-butylamine, n-octylamine or phenethylamine, and is added in limited quanity of the activated support of Formula III followed by an excess of tris (hydroxymethyl)-aminomethane in aqueous solution, a support suitable for hydrophobic interaction chromatography of biological macromolecules of Formula VII results.

If the functionalizing reagent is a diamine, aminoalkylcarboxylic acid or short peptide of general formula RNH_2, such as 1,6-diaminohexane, 4-aminobutyric acid, 6-aminohexanoic acid or diglycine, and is added in limited quantity to the activated support of Formula III followed by an excess of tris (hydroxymethyl) aminomethane in aqueous solution, a support suitable for affinity chromatography of biological macromolecules of Formula VII results in which R contains an amine or carboxylic acid group through which a ligand or protein can be immobilized.

VII



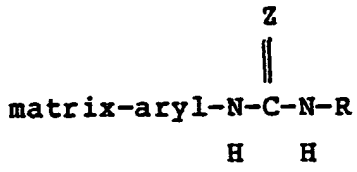
Imidodiacetic acid can be used as a functionalizing agent thereby creating activated support having the form as follows, for use as a metal chelator.



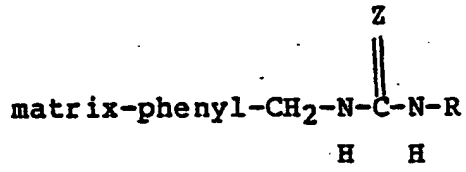
A variety of azolides other than N,N'-carbonyldiimidazole may be employed in preparation of the activated support, Formula III. Such alternatives include N,N'-carbonyldipyrazole, N,N'-carbonyldi-1,2,3-triazole, N,N'-carbonyldi-1,2,4-triazole, N,N'-carbonyldiindole, N,N,-carbonylidibenzimidazole and N,N'-carbonyldibenztriazole and others.

Alternatively, N,N'-thiocarbonyldiimidazole or thione analogs of the compounds listed above may also be employed to prepare thio-urea analogs of the various types described by this invention. Materials of this type are believed to exhibit physical properties very similar to the urea derivatives and would also represent advantages over prior art.

In place of the alkyl chain support described above having the form $(CH_2)_n$ the present invention can also be applied to polymeric chromatographic support, wherein an aryl replaces the alkyl chain, as shown below.



Specifically, a compound having the following form may be employed.



Example I

N-Carboxymethyl-N'-propylsilylurea Silica
Preparative Ion Exchange Chromatography

Aminopropyl silica gel (1.5% N), 40 uM irregular with 60 Angstron average porosity, is dried at 80°C for three hours then allowed to cool to room temperature in a desiccator. For each gram of aminopropyl silica gel, 0.8 grams of N,N'-carbonyl-diimidazole and 0.13 milliliters of triethylamine are dissolved in 10 milliliters of methylene chloride. The aminopropyl silica gel is added to the reaction mixture and stirred for three hours at room temperature. The activated silica gel is filtered from solution and washed with methylene chloride, dioxane, 1:1 dioxane:water and twice with water. The activated silica gel is then immediately added to a solution of 4% (w/v) glycine in 0.1 N sodium carbonate buffer. For each gram of activated silica gel, 10 milliliters of solution is employed. The reaction mixture is stirred for 24 hours at room temperature. The product is filtered from solution and washed with water, 0.1 N hydrochloric acid, 1 N sodium chloride and twice with water. The product is then allowed to dry to room temperature.

Example II

N-Tris(hydroxymethyl)-N'-propylsilylurea Silica Size Exclusion Chromatography of Biological Macromolecules

Aminopropyl silica gel (0.5% N), 5 uM spherical with 300 Angstron average porosity, is dried at 80°C for three hours then allowed to cool to room temperatur in a desiccator. For each gram of aminopropyl silica gel, 0.3 grams of N,N'-carbonyldiimidazole and 0.05

milliliters of triethylamine are dissolved in 8 milliliters of tetrahydrofuran (THF). The aminopropyl silica gel is added to the reaction mixture and stirred for three hours at room temperature. The activated silica gel is filtered from solution and washed with THF and twice with water. The activated silica gel is then immediately added to a solution of 0.5 N tris (hydroxymethyl) aminomethane, pH 9.3. For each gram of activated silica gel, 8 milliliters of solution is employed. The reaction mixture is stirred for 24 hours at room temperature. The product is filtered from solution and washed with water, 0.1 N hydrochloric acid, 1 N sodium chloride and twice with water. The product is then allowed to dry to room temperature.

Example III

N-Phenylmercury-N'-propylsilyurea Silica Preparative Covalent Chromatography

Aminopropyl silica gel (1.5% N), 40 uM irregular 60 Angstron average porosity, was dried at 80 C for three hours then allowed to cool to room temperature in a desiccator. For each gram of aminopropyl silica gel, 0.8 grams of N,N'-carbonyldiimidazole and 0.13 milliliters of triethylamine are dissolved in 10 milliliters of methylene chloride. The aminopropyl silica gel is added to the reaction mixture and stirred for three hours at room temperature. The activated silica gel is filtered from solution and washed with methylene chloride and twice with dimethylsulfoxide (DMSO). The activated silica gel is then immediately added to a solution of 1% p-aminophenylmercuric acetate in 90% DMSO. For each gram of activated silica gel, 7 milliliters of solution is

employed. The reaction mixture is stirred for 24 hours at 40 C filtered from solution and then returned to a solution of ammonia saturated DMSO and the reaction mixture stirred for 3 hours at room temperature. Finally, the product is filtered from solution and washed with 50% DMSO, water, 0.1 N hydrochloric acid, 1 N sodium chloride and twice with water. The product is then allowed to dry to room temperature.

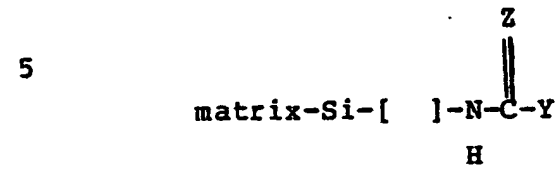
Example IV

N-Butyl-N'-propylsilylurea Silica Hydrophobic Interaction Chromatography of Biological Macromolecules

Aminopropyl silica gel (0.5%N), 5 uM spherical with 300 Angstron average porosity, was dried at 80 C for three hours then allowed to cool to room temperature in a desiccator. For each gram of aminopropyl silica gel, 0.3 grams of N,N'-carbonyldiimidazole and 0.5 milliliters of triethylamine are dissolved in 8 milliliters of tetrahydrofuran (THF). The aminoproply silica gel is added to the reaction mixture and stirred for 3 hours at room temperature. The activated silica gel is filtered from solution and washed with THF and twice with water. The activated silica gel is then immediately added to a solution 6% n-butylamine in 0.1 N sodium carbonate. For each gram of activated silica gel, 9 milliliters of solution is employed. The reaction mixture is stirred for 24 hours at room temperature. The modified silica gel is then filtered from solution, washed twice with water, and returned to a solution of 0.5 N tris (hydroxymethyl) aminomethane, pH 9.3, and the reaction mixture stirred for 3 hours at room temperature. Finally, th product is filtered from soluti n and washed with water, 0.1 N hydrochloric acid, 1 N sodium chloride and twice with wat r. The product is then allowed to dry to ro m temperature.

Claims

1. A compound of general Formula I



wherein matrix is particulate bonded silica or controlled pore glass beads, Y is an azole or derivative, Z is oxygen or sulfur, and [ ] is a support selected from a straight chain alkyl having a chain length of 2 to 8 carbons, an aryl, alkyl, or alkyl aryl.

2. The compound of Claim 1 wherein said azole is selected from imidazoyl, triazoyl, benzimidazoyl, benztriazoyl, indolyl and pyrazolyl.

3. The compound of Claim 2 wherein said azole is selected from imidazoyl and triazoyl.

4. A compound having the general Formula II

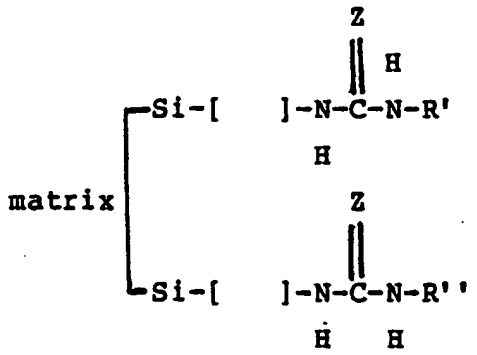
where R is selected from hydrogen, ethylsulfonic acid, carboxymethyl, diethylaminoethyl, and guanidino;

Z is oxygen or sulfur;

[ ] is a support selected from a straight chain alkyl having 2 to 8 carbons, an alkyl, aryl, or alkyl aryl; and

matrix is particulate bonded silica or controlled pore glass beads.

5. The compound having the general Forumla III,



III

wherein R' is selected from hydrogen, urea and tris (hydroxymethyl);

R'' is selected from the group consisting of hydrogen, ethylsulfonic acid, carboxymethyl, diethylaminoethyl, guanidino, phenylboronic acid, phenylmercuric acetate, phenylarsineoxide, tris (hydroxymethyl), n-butyl, n-octyl, hexanoic acid and ethylmercapto;

matrix is particulate bonded silica or controlled pore glass beads;

Z is oxygen or sulfur; and

[ ] is a support selected from a straight chain alkyl having 2 to 8 carbons, an aryl, or alkyl aryl.

6. The compound of Claim 5 wherein the ratio of R' to R'' is predetermined.

7. The compound of Claim 5 wherein R'' comprises phenylboronic acid.

8. The compound of Claim 5 wherein R'' comprises phenylmercuric acetate.

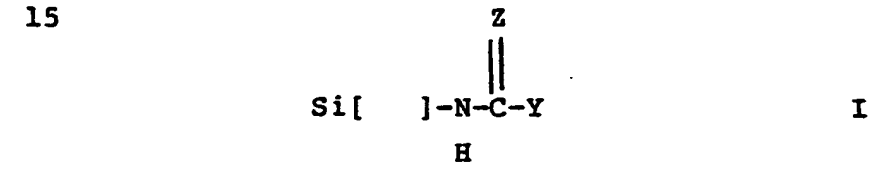
9. The compound of Claim 5 wherein R' and R'' both comprise N,N-di (carboxymethyl).

10. The compound of any of Claims 1-9, wherein said compound is packed in a column.

11. The compound of any of Claims 1-9 wherein the functional groups on said supports are 20% to 30% derivatized, and the remainder of the functional groups on said supports are inactivated with hydrogen, ethanol or tris (hydroxmethyl).

12. The compound of any of Claims 1, 4, 5, 14, 16 and 17 wherein said alkyl chain has 3 or 4 carbons.

13. A process for the preparation of a compound of general Formula I



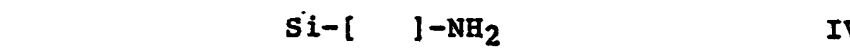
I

where [ ] is a support selected from a straight chain alkyl having 2 to 8 carbons, an alkyl, aryl, or alkyl aryl, and

Z is oxygen or sulfur; and

Y is an azole or derivative;

comprising reacting a compound of general Formula IV



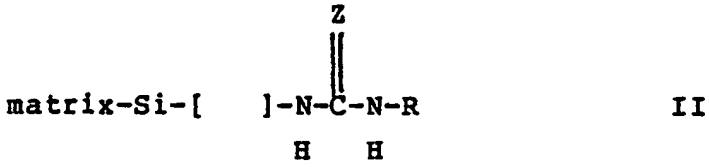
IV

with an azolide in anhydrous organic solvent at room temperature or at elevated temperature for at least several minutes.

14. The process of Claim 13 wherein said azolide is selected from N,N'-carbonyldiimidazole, N,N'-carbonyldipyrazole, N,N'-carbonyldi-1,2,3-triazole, N,N'-carbonyldi-1,2,4-triazole, N,N'-carb nyldiindole, N,N'-carbonylidibenzimidazole and N,N'-carbonyldibenztriazole.

15. The process of Claim 14 wherein said azolide is N,N'-carbonyldiimidazole.

16. A process for the preparation of a compound of general Formula II



II

where R is selected from hydrogen, ethylsulfonic acid, carboxymethyl, diethylaminoethyl, and guanidino,

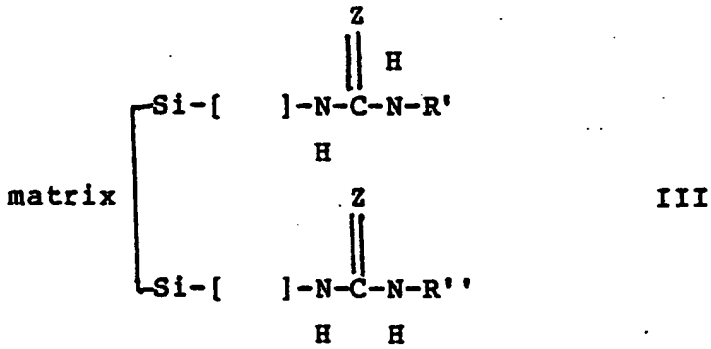
Z is oxygen or sulfur,

[ ] is a support selected from a straight chain alkyl having 2 to 8 carbons, an alkyl, aryl, or alkyl aryl, and

matrix is particulate bonded silica or controlled pore glass beads;

comprising adding a primary or secondary alkyl, aryl, or alkyl aryl amine where R is selected from hydrogen, ethylsulfonic acid, carboxymethyl, diethylaminoethyl, and guanidino in an organic solvent or an aqueous solution of said amine or its salt.

17. A process for the preparation of a compound of general Formula III



III

where matrix is particulate b nded silica or controlled pore glass beads;

Z is oxygen or sulfur; and

[ ] is a support selected from a straight chain alkyl having 2 to 8 carbons, an aryl, or alkyl aryl;

comprising adding a primary or secondary alkyl or aryl amine of the formula HNR'R'', where R' and R'' are selected from H, alkyl, aryl or derivative thereof, in an organic solvent or an aqueous solution of said amine or its salt.

18. The process of Claim 17 wherein R' is selected from hydrogen, urea and tris (hydroxymethyl); and

R'' is selected from the group consisting of hydrogen, ethylsulfonic acid, carboxymethyl, diethylaminoethyl, guanidino, phenylboronic acid, phenylmercuric acetate, phenylarsineoxide, tris (hydroxymethyl), n-butyl, n-octyl, hexanoic acid and ethylmercapto.

19. The process of Claims 17 or 18 wherein said R' is added in less than stoichometric amounts and is followed by the addition of an excess of said R''.

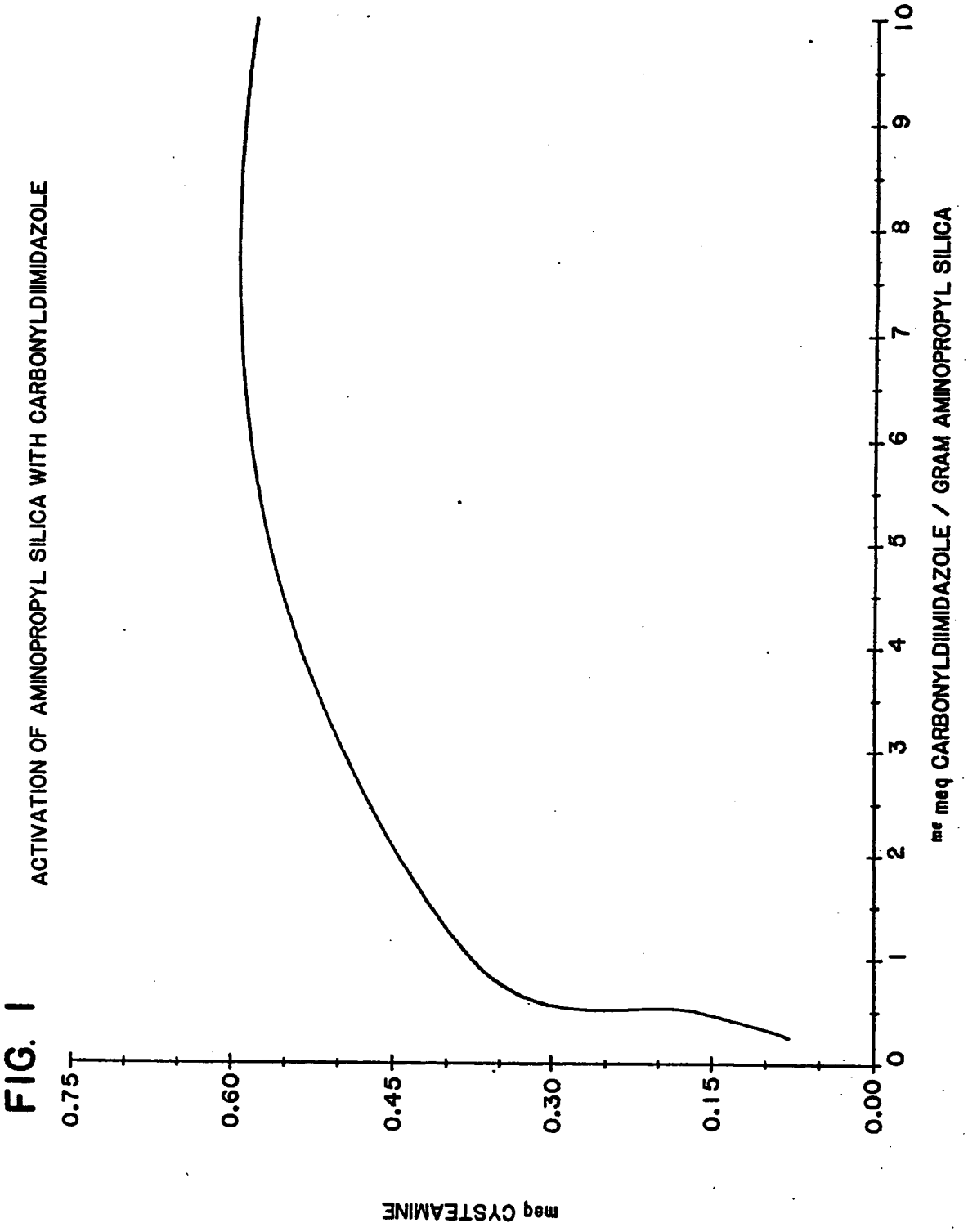

ACTIVATION OF AMINOPROPYL SILICA WITH CARBONYLDIIMIDAZOLE
mg CYSTEAMINE
0.75
0.60
0.45
0.30
0.15
0.00
0
1
2
3
4
5
6
7
8
9
10
m² meq CARBONYLDIIMIDAZOLE / GRAM AMINOPROPYL SILICA

FIG. 1

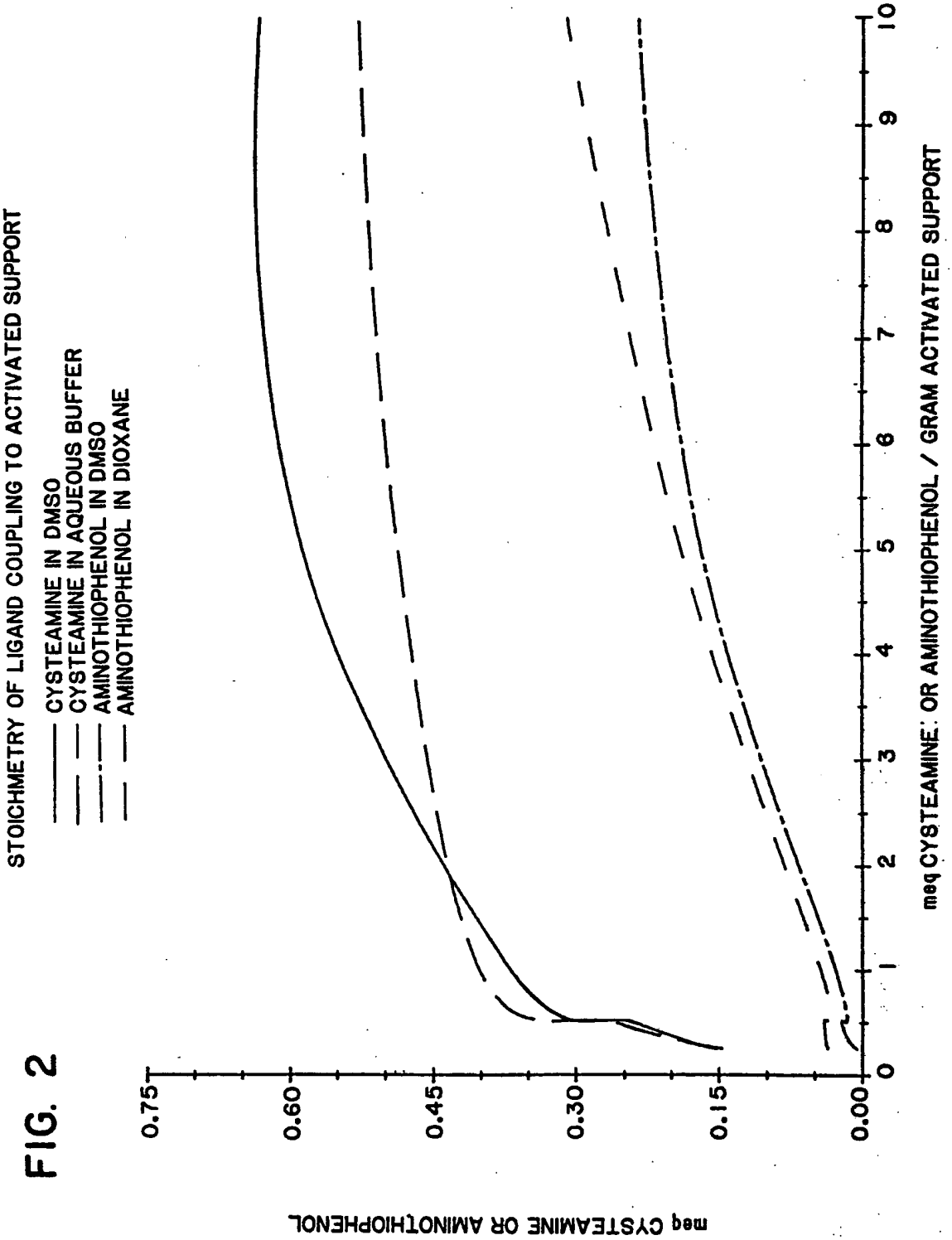

STOICHMETRY OF LIGAND COUPLING TO ACTIVATED SUPPORT
CYSTEAMINE IN DMSO
CYSTEAMINE IN AQUEOUS BUFFER
AMINOTHIOPHENOL IN DMSO
AMINOTHIOPHENOL IN DIOXANE
meq CYSTEAMINE OR AMINOTHIOPHENOL
0.75
0.60
0.45
0.30
0.15
0.00
0
1
2
3
4
5
6
7
8
9
10
meq CYSTEAMINE OR AMINOTHIOPHENOL / GRAM ACTIVATED SUPPORT

FIG. 2

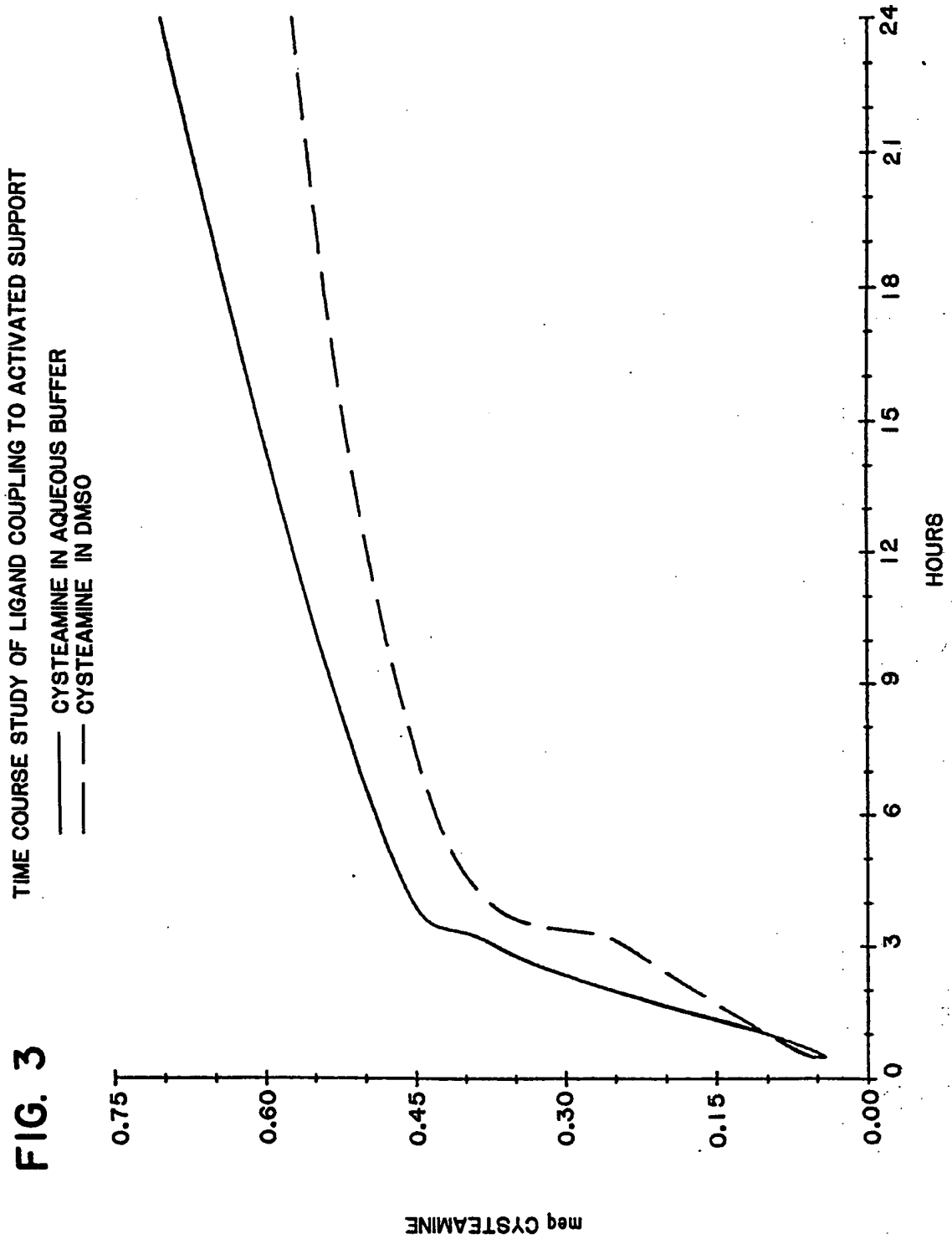

TIME COURSE STUDY OF LIGAND COUPLING TO ACTIVATED SUPPORT
CYSTEAMINE IN AQUEOUS BUFFER
CYSTEAMINE IN DMSO
meq CYSTEAMINE
0.75
0.60
0.45
0.30
0.15
0.00
0
3
6
9
12
15
18
21
24
HOURS

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00901

I. CLASSIFICATI N F SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07F 7/10
U.S. CL: 548/110; 556/9, 70, 402, 421

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	548/110; 556/9, 70, 402, 421

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CAS ONLINE: STRUCTURE SEARCH

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US,A, 2,907,782 (PIKE) 06 October 1959. See column 1, lines 22-36.	4-9
A	US,A, 4,316,041 (TOTTEN ET AL) 16 February 1982. See column 2, lines 1-63.	4-9
X	US,A, 4,435,567 (LUGOSI ET AL) 6 March 1984. See column 1, lines 19-56 and column 2, lines 19-33.	16-19

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
19 Jun 1987	30 JUN 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Richard L. Raymond Richard L. Raymond

Form PCT/ISA/210 (second sheet) (May 1986)